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Both recombinant African catfish LH and FSH are able to activate the African catfish FSH receptor

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Abstract

LH and FSH are heterodimeric glycoprotein hormones, composed of a common α-subunit non-covalently associated with a hormone-specific β-subunit. Repeated efforts to isolate catfish FSH (cfFSH) have not been successful and only catfish LH (cfLH) has been purified from catfish pituitaries. Recently, however, we succeeded in cloning the cDNA encoding the putative cfFSHβ; the cDNAs for the α- and β-subunit of cfLH have been cloned before.

Here we report the expression of biologically active cfLH and cfFSH in the soil amoeba, *Dictyostelium discoideum*. The biological activity of the recombinant hormones was analyzed using cell lines transiently expressing either the cfLH receptor or the cfFSH receptor. Moreover, a primary testis tissue culture system served to study the steroidogenic potency of the recombinant hormones.

Our results demonstrated that *Dictyostelium* produced biologically active, recombinant catfish gonadotropins, with recombinant cfLH being almost indistinguishable from its native counterpart, purified from pituitaries. Although recombinant cfFSH has significant effects in the bioassays used in this study, the specific function of native cfFSH in the control of reproduction and its expression patterns are not yet understood.

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Introduction

The pituitary-derived gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), play pivotal roles in the regulation of vertebrate reproduction by stimulating the gonads via their respective membrane-inserted receptors. In the mammalian testis, the gonadotropin–gonadotropin receptor interactions are highly specific (Braun *et al.* 1991, Tilly *et al.* 1992): LH stimulates androgen production by the Leydig cells, expressing the LH receptor (LH-R), whereas FSH acts on the germ cell-supporting Sertoli cells, expressing the FSH receptor (FSH-R), resulting in an increased efficiency of androgen-dependent spermatogenesis (McLachlan *et al.* 1996). Hence, the combined complementary actions of both LH and FSH are required for efficient spermatogenesis (Themmen & Huhtaniemi 2000).

The above-described functional duality of gonadotropins is less obvious in teleosts. Salmon FSH, the steroidogenic activity of salmon FSH and salmon LH were similar (Planas & Swanson 1995). In addition, salmon FSH can activate both the salmon FSH-R and LH-R (Oba *et al.* 1999*a*, *b*). Such promiscuous hormone–receptor interaction has also been observed in African catfish: catfish LH (cfLH) can stimulate both catfish FSH-R (cfFSH-R)- as well as catfish LH-R (cfLH-R)-mediated intracellular cAMP production with efficacies that are in the same order of magnitude (Bogerd *et al.* 2001, Vischer & Bogerd 2003).

There is no functional information available for catfish FSH (cfFSH), as the isolation of native cfFSH has failed repeatedly. However, we recently succeeded in cloning the putative cfFSHβ cDNA (Vischer *et al.* 2003). This provided the tools to produce cfFSH, which can be used for experimentation in the absence of purified pituitary cfFSH, in a heterologous expression system. Moreover, recombinant preparations of cfFSH (rcfFSH) will not be contaminated with closely related hormones

like cfLH or catfish thyrotropin that may interfere with the analysis of the ligand-binding properties of fish gonadotropin receptors. The cDNAs encoding the α- and β-subunits of cfLH have been cloned previously (Rebers *et al.* 1997).

In the present study, we describe the expression of recombinant cfLH (rcfLH) and rcfFSH in the slime mold, *Dictyostelium discoideum*. The bioactivities of both recombinant catfish gonadotropins were analyzed with respect to their potency to stimulate testicular steroidogenesis in a short-term tissue culture system, as well as cAMP production in cells transiently expressing the cfFSH-R or the cfLH-R. The data obtained were compared with the activities of native cfLH, purified from catfish pituitaries (Schulz *et al.* 2001).

Materials and methods

Animals

African catfish (*Clarias gariepinus*) were bred and raised in the laboratory as previously described (De Leeuw *et al.* 1985), except that catfish pituitary extract instead of human chorionic gonadotropin (hCG) was used to induce ovulation. Animal culture and experimentation were consistent with the Dutch national regulations; experimental protocols were submitted to and approved by the respective University committee. For this study, testes of sexually mature, adult 1·5-year-old males were used.

Gonadotropins

cfLH was isolated from pituitaries of mature catfish as described (Schulz *et al.* 2001).

Expression vectors and cDNA cloning

In order to facilitate highly efficient mRNA translation (initiation) in the *Dictyostelium discoideum* expression system, a *Dictyostelium* consensus translation initiation sequence (AAAAA; Vervoort *et al.* 2000) was introduced in front of the translation initiation codon (ATG) of each of the catfish glycoprotein α-subunit (cfGPα), cfLH β-subunit (cfLHβ) and cfFSH β-subunit (cfFSHβ) cDNAs. Moreover, the first 30 bases coding for each of the three catfish gonadotropin-subunit coding sequences were altered to the preferred *Dictyostelium* codon usage (Vervoort *et al.* 2000) using a PCR-based mutagenesis approach. To this end, the cfGPα cDNA (Rebers *et al.* 1997), and the cfLHβ and cfFSHβ cDNAs (Rebers *et al.* 1997, Vischer *et al.* 2003) were used as templates for PCR. In addition, BglII and AatII restriction endonuclease sites were introduced at the 5′- and 3′-ends respectively of the cfGPα cDNA sequence to facilitate directional subcloning in the *Dictyostelium* extra-chromosomal, blasticidin-resistant expression vector MB12n (Heikoop *et al.* 1998), yielding the cfGPα/MB12n construct, in the PCR-based mutagenesis step, described above. In a similar way, BglII and SpeI restriction endonuclease sites were introduced at the 5′- and 3′- end respectively of both the cfLHβ and cfFSHβ cDNA sequences to facilitate directional subcloning in the *Dictyostelium* extra-chromosomal, neomycin-resistant expression vector MB12 neo (Linskens *et al.* 1999), yielding the cfLHβ/MB12 neo and cfFSHβ/MB12 neo constructs respectively. To this end, we used the following primers obtained from Life Technologies (Breda, The Netherlands): GPα-sense, 5′-TA*AGA TCT***AAAAA**-ATGACTTTAATTCCAAAATACA CTGGTGCAACAATTCTTTTACTG-3′; GPα-antisense, 5′-TA*GACGTC*TAAAACTTATGATA GTAGCAAGTGCT-3′; FSHβ-sense, 5′-TA*AGA TCT***AAAAA**-ATGATGATGCGTGGTGTTGCT ATGGTTTTGCTCTTGCCAATG-3′; FSHβ-antisense, 5′-TA*ACTAGT*GGCTGAGCTGATGT GACTC-3′; LHβ-sense, 5′-TA*AGATCT***AAAAA**-ATGCCAGCTTCATCATATTTTTTATTATT CTTCTTTATGAAC-3′; LHβ-antisense, 5′-CAT GGC*ACTAGT*TCAATAGTCCAGAATCCCCTC TT-3′; in which all sense primers contained a *Dictyostelium* consensus translation initiation sequence (bold uppercase) and *Dictyostelium* codon usage adaptations (underlined), while restriction endonuclease sites are represented in italics.

Expression of recombinant hormones

Dictyostelium discoideum (strain AX3) was grown in axenic medium essentially as described by Watts & Ashworth (1970), with 10 g/l glucose instead of 15·4 g/l. Approximately 10^7 cells were co-transformed with 1 μg cfGPα/MB12n, and 1 μg cfLHβ/MB12 neo or cfFSHβ/MB12 neo, by electroporation, as previously described (Howard *et al.* 1988, Heikoop *et al.* 1998). Next, the cells were seeded in 9 cm culture plates. The selection of cells

harboring the cfGPα/MB12n plasmid was started 5 h after electroporation by adding blasticidin S (10 μg/ml; ICN, Zoetermeer, The Netherlands). After 24 h, the medium was aspirated, and fresh medium containing 10 μg/ml blasticidin S and 5 μg/ml neomycin G418 (Invitrogen, Breda, The Netherlands) was added, thus selecting cells harboring the cfGPα/MB12n plasmid in combination with either the cfLHβ/MB12 neo or the cfFSHβ/MB12 neo plasmid. Medium was replaced every 3–4 days, while maintaining selective conditions. After 10–14 days, the positive transformants had grown to confluency. The cells were maintained in a 9 cm plate and medium (∼6 ml) was harvested every 3·5 days, in total three or four times. During each harvesting, about half of the *Dictyostelium* cells were removed from the plate allowing the remaining cells, after addition of fresh medium (containing blasticidin S and neomycin G418), to grow again from 50 to 100% confluency. The harvested medium was stored at −20 °C until analysis.

rcfLH and rcfFSH were ∼20-fold concentrated, while simultaneously replacing the *Dictyostelium* growth medium by DMEM–Hepes (Sigma, St Louis, MO, USA), by ultrafiltration using Ultrafree-15 Biomax-30 centrifugal filter devices (Amicon; Millipore Corporation, Bedford, MA, USA). Recombinant catfish gonadotropins were quantified by RIA using antisera against intact cfLH and the cfGPα-subunit, as described previously (Schulz *et al.* 1995).

In vitro receptor activation

Ligand-stimulated cAMP production, mediated via either the cfFSH-R or the cfLH-R, was determined as described previously (Bogerd *et al.* 2001, Vischer & Bogerd 2003). Briefly, approximately 5×10^6 human embryonic kidney T 293 (HEK-T 293) cells were co-transfected with 10 μg of a reporter construct with a β-galactosidase gene driven by five cAMP responsive elements (Chen *et al.* 1995) and either 1 μg cfFSH-R or 10 ng cfLH-R expression vector constructs. One hundred-fold less expression vector construct of the constitutively active cfLH-R was used for transfection, compared with cfFSH-R; this reduces the cfLH-R-mediated basal cAMP levels allowing the dose–response curves to fit within the measuring range of the reporter gene assay (Vischer & Bogerd 2003). After 16–18 h, the cells were collected and transferred to 96-well plates ($\sim 2{\cdot}5 \times 10^5$ cells/well). The next day, the transfected cells were stimulated for 6 h with rcfFSH, rcfLH, or purified, pituitary-derived cfLH in 25 μl DMEM–Hepes containing 0·1% BSA (both from Sigma). Ligand-induced β-galactosidase activities were measured, and the hormone concentrations inducing a half-maximal stimulation (EC_{50}) were calculated using the Graphpad PRISM3 software package version 3·00 (GraphPad Software, Inc., San Diego, CA, USA). All experiments were repeated at least three times using cells from independent transfections and two different batches of recombinant hormones.

11β-Hydroxyandrostenedione (OHA) secretion by African catfish testis *in vitro*

Testicular tissue was prepared for *in vitro* incubations as described previously (Schulz *et al.* 1994) with the following modifications. Testis tissue from five adult males was pooled in a Petri dish containing DMEM–Hepes (Sigma) supplemented with L-glutamine (8 mM), and penicillin G and streptomycin (100 U/ml each; all from Invitrogen). While submerged in medium, the tissue was minced into fragments of $\sim 3\ mm^3$. The tissue fragments were filtered over two layers of cheesecloth, pre-incubated in fresh medium in a shaking incubator at 25 °C for 30 min, and filtered again over two layers of cheesecloth, to remove suspended sperm and tissue debris. Four or five randomly selected fragments (total wet weight 15–20 mg) were placed per well of a 48-well culture plate containing 0·2 ml medium. Due to the limited availability of rcfFSH and rcfLH as well as highly purified, pituitary-derived cfLH, five replicates per ligand concentration were tested in dose–response experiments. The tissue fragments were incubated at 25 °C for 18 h in the presence of varying concentrations of recombinant and native hormones, as indicated. The incubation medium was then collected, heated at 80 °C for 1 h and centrifuged at 16 000 ***g*** at room temperature for 30 min. The supernatants were stored at −20 °C, until the secreted OHA (4-androsten-11β-ol-3,17-dione) levels were determined by RIA, as described previously (Schulz *et al.* 1996). OHA is the quantitatively dominating androgen secreted by African catfish testis (Vermeulen *et al.* 1993). The results are expressed as ng OHA secreted/mg testis tissue.

Table 1 Amount of recombinant catfish gonadotropins/ml medium, after ~20-fold concentration by ultrafiltration, as quantified by RIA using antisera against intact cfGPα-subunit and intact cfLH, as described by Schulz *et al*. (1995)

	cfGPα measured (ng/ml)	**Intact recombinant hormone calculated**[b] (ng/ml)	**Intact cfLH measured** (ng/ml)
Hormone[a]			
rcfLH(1)	455	1002	1058
rcfLH(2)	688	1513	1487
rcfLH(3)	347	763	733
rcfFSH(1)	475	1045	86
rcfFSH(2)	188	415	56
rcfFSH(3)	410	903	n.d.[c]

[a]Different batches of recombinant hormones are indicated by the numbers in brackets.
[b]Intact recombinant hormone concentrations were calculated from the anti-GPα measurements, which were multiplied by 2·2.
[c]Not determined.

Statistics

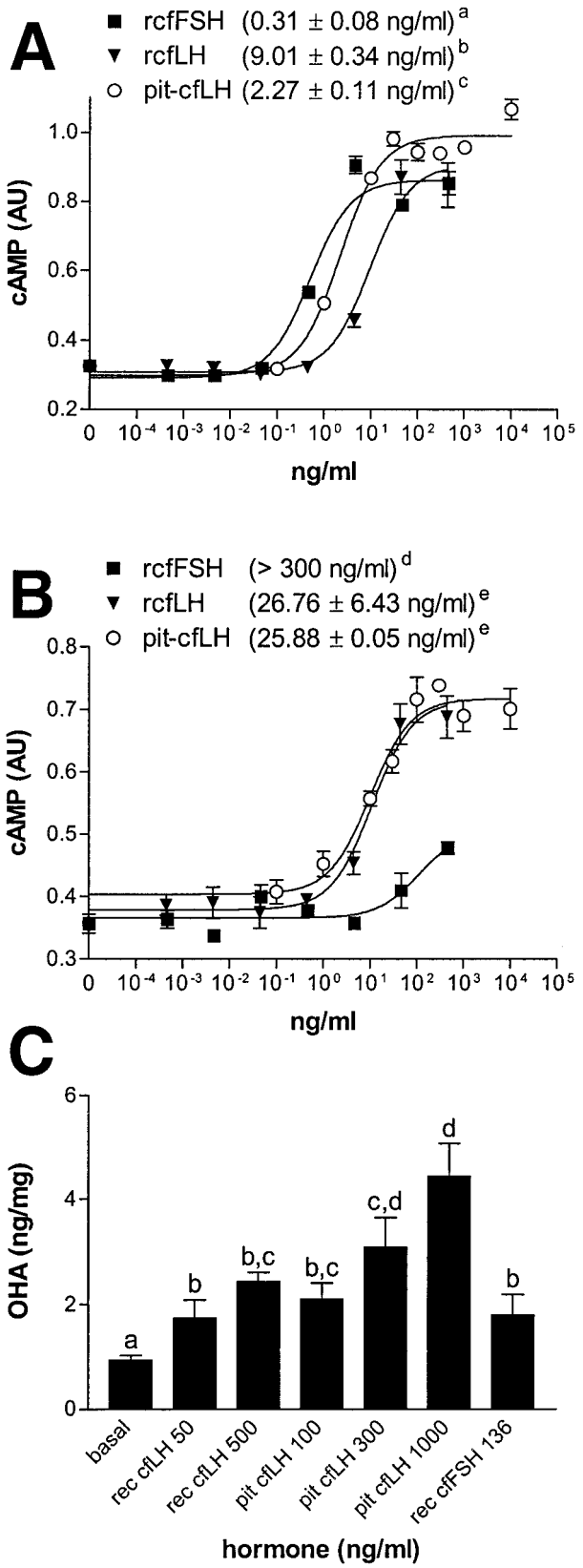
All results are expressed as the means ± S.E.M. To evaluate the effect of increasing hormone concentrations between transfected cells and testis fragments, the β-galactosidase activity and OHA production respectively were subjected to ANOVA using Statview 4·5 (Abacus Concepts, Berkeley, CA, USA). OHA medium levels were $\log_{10}$ transformed prior to analysis in order to obtain equal residual variation among treatments. The ANOVA was followed by a Fisher's probable least-squares difference (PLSD) test to identify significant differences ($P < 0{\cdot}05$) between individual groups. The same analysis was used to compare half-maximal stimulation (EC_{50}) of the cfFSH-R- or cfLH-R-mediated signal transduction, induced by the purified, pituitary-derived cfLH, and rcfLH and rcfFSH.

Results

Catfish gonadotropin expression in *Dictyostelium*

Dictyostelium discoideum, co-transformed with the cfGPα/MB12n plasmid, and either the cfLHβ/MB12 neo or the cfFSHβ/MB12 neo plasmid, synthesized and secreted rcfLH or rcfFSH respectively into the medium when cultured under double selective (i.e. blasticidin S and neomycin G418) conditions. Two weeks after co-transformation, approximately 6 ml medium per plate were harvested at ~3·5 day intervals, in total three or four times. The axenic *Dictyostelium* culture medium was substituted by DMEM–Hepes and simultaneously concentrated to a volume of ~480 µl using Ultrafree-15 Biomax-30 centrifugal filter devices. The recombinant hormones were first functionally analyzed (see below), and *a posteriori* quantified by RIA in order to limit the storage time between the concentration procedure and the functional assays.

When rcfLH amounts were analyzed using the specific cfGPα RIA, ~2·2 ± 0·11 ($n = 3$)-fold lower levels were found than when using the RIA specific for intact cfLH (Table 1). This corresponds to cfGPα contributing approximately half of the total molecular mass of intact heterodimeric cfLH (Koide *et al.* 1992, Rebers *et al.* 1997). Since neither specific antiserum nor purified hormone was available for developing a specific cfFSH RIA, the rcfFSH levels were calculated from the anti-GPα measurements. Also here the factor 2·2 was used since the mass ratio predicted from the nucleotide sequences are very similar for cfFSHβ/GPα and cfLHβ/GPα (data not shown). When comparing calculated values with the immunological activity of rcfFSH in the intact-cfLH RIA (Table 1), only about 11% of the calculated rcfFSH levels were detected. For convenience, the calculated recombinant catfish gonadotropin levels were quantified based on the cfGPα RIA data for an estimation of the production of recombinant proteins. This showed that the *Dictyostelium* cells in a single 9 cm plate produced 18–61 ng rcfFSH and 31–80 ng rcfLH per ml of medium. These amounts are very



similar to the production levels of recombinant human gonadotropins by *Dictyostelium* cells (Linskens *et al.* 1999).

Bioactivities of the recombinant catfish gonadotropins

The bioactivities of both recombinant catfish gonadotropins were analyzed by determining their capacity to stimulate intracellular cAMP production in HEK-T 293 cells transiently expressing the cfFSH-R or the cfLH-R, as well as their capacity to stimulate OHA production by testis tissue fragments in primary culture. Both recombinant gonadotropins were able to activate the cfFSH-R (Fig. 1A): rcfFSH was ~7-fold more bioactive (EC_{50}=0·31 ± 0·08 ng/ml) than the purified, pituitary-derived cfLH (EC_{50}=2·27 ± 0·11 ng/ml), while rcfLH was significantly less potent (EC_{50}= 9·01 ± 0·34 ng/ml) than its purified, pituitary-derived counterpart. Equal efficacy was observed between rcfLH (EC_{50}=26·76 ± 6·43 ng/ml) and purified, pituitary-derived cfLH (EC_{50}=25·88 ± 0·05 ng/ml) with respect to cfLH-R activation (Fig. 1B). Also rcfFSH was able to stimulate the cfLH-R-mediated cAMP production significantly, but with a much lower efficacy (EC_{50} estimated to be >300 ng/ml) than purified cfLH.

Incubation of testis tissue fragments pooled from five mature male African catfish with rcfFSH

Figure 1 Bioactivities of purified, pituitary-derived cfLH as well as rcfLH and rcfFSH were determined on HEK-T 293 cells transiently expressing the cfFSH-R or the cfLH-R, and on African catfish testis tissue fragments. (A) Effects of native cfLH and both recombinant catfish gonadotropins on HEK-T 293 cells transiently co-transfected with the cfFSH-R and a cAMP-responsive reporter gene construct. (B) Effects of native cfLH and both recombinant catfish gonadotropins on HEK-T 293 cells transiently co-transfected with the cfLH-R and a cAMP-responsive reporter gene construct. Numbers in brackets represent EC_{50} values, representing the average of at least three independent assays with triplicate observations each, and are given as means±S.E.M. Values sharing the same letter do not significantly differ (P<0·05; ANOVA, followed by Fisher's PLSD test). AU=arbitary units. (C) Effects of native cfLH and both recombinant catfish gonadotropins on androgen (OHA; see text) production by African catfish testis tissue fragments *in vitro*. All data are shown as means±S.E.M. of triplicate observations from a single representative experiment. Treatments sharing the same letter did not significantly differ (P<0·05; ANOVA followed by Fisher's PLSD).

(136 ng/ml) and rcfLH (50 and 500 ng/ml) resulted in a significant *in vitro* secretion of OHA over basal (Fig. 1C). Within the tested range the efficacy of rcfLH (50 and 500 ng/ml) was comparable with that of pituitary-derived cfLH (100 and 300 ng/ml).

Discussion

Under physiological conditions, the two mammalian gonadotropins LH and FSH interact specifically with their respective cognate receptors, since a 50- to 100-fold excess of the heterologous hormone is required to cross-activate these receptors (Braun *et al.* 1991, Tilly *et al.* 1992). Moreover, in mammalian testis, the LH-R is only expressed on Leydig cells, and the FSH-R only on Sertoli cells. Thus, stimulation of the testis with (each of) the gonadotropins results in the specific activation of Leydig cell steroidogenesis by LH, and in supporting spermatogenesis, mediated by the specific stimulation of Sertoli cell functions, by FSH (Zirkin 1998).

In teleosts, however, this functional duality of gonadotropins seems to be less apparent. For example, both salmonid gonadotropins stimulated testicular steroidogenesis *in vitro* with equal potencies (Planas & Swanson 1995). Studies on cloned amago salmon LH-R and FSH-R provided additional evidence for promiscuous hormone–receptor interactions, as salmon FSH (5 μg/ml) was able to interact with both receptors (Oba *et al.* 1999*a*,*b*); however, other hormone concentrations have not been tested.

Hitherto, only cfLH, but not cfFSH, has been purified from pituitaries of the African catfish (Koide *et al.* 1992, Schulz *et al.* 2001). cfGPα and cfLHβ mRNA as well as the heterodimeric hormone are present in the pituitary, and gonadotropin-releasing hormone-induced release of cfLH from the pituitary has been demonstrated, at all sexual developmental stages (Schulz *et al.* 1995, 1997). Purified, pituitary-derived cfLH stimulates intracellular cAMP production in HEK-T 293 cells expressing either the cfLH-R (Vischer & Bogerd 2003) or the cfFSH-R (Bogerd *et al.* 2001) with comparable efficacies, as compared with the situation in mammals. Taken together, these data open the possibility that cfLH may cover gonadotropin-regulated functions that are usually separated, and require the complementary activity of both FSH and LH. Notwithstanding this possibility, phylogenetic considerations make it reasonable to assume that teleost fish, like tetrapod vertebrates, express both an FSH- and an LH-type of gonadotropin (Quérat *et al.* 2001). It may therefore not be surprising that we recently identified the gene encoding the putative cfFSHβ, and confirmed the presence of its mRNA in the pituitary (Vischer *et al.* 2003).

In order to get more insight into potential physiological function(s) of cfFSH in African catfish, we decided to produce recombinant cfFSH using the soil amoeba, *Dictyostelium discoideum*. This organism has proven to be an attractive alternative for CHO cells for the heterologous expression of glycoprotein hormones (Heikoop *et al.* 1998, Linskens *et al.* 1999), as it performs several mammalian-like post-translational modifications (e.g. glycosylation) and is able to secrete human FSH and hCG with bioactivities comparable with their CHO cell-produced counterparts (Heikoop *et al.* 1998, Linskens *et al.* 1999). In addition, *Dictyostelium* is more easy to transform and to grow (Linskens *et al.* 1999).

rcfLH, produced in *Dictyostelium*, had approximately the same bioactivity as the purified, pituitary-derived cfLH, regarding the stimulation of cfLH-R-mediated cAMP production in transiently transfected HEK-T 293 cells as well as the stimulation of testicular androgen production. In contrast, rcfLH was slightly less active than the purified, pituitary-derived cfLH in stimulating the cfFSH-R. This may be due to the presence of residual cfFSH in the purified, pituitary-derived cfLH material, which cannot be immunologically detected since no cfFSH-specific antibody is available yet. However, biochemical analyses revealed no evidence for such a contamination (Schulz *et al.* 2001). rcfFSH was ~7-fold more bioactive than purified, pituitary-derived cfLH, in stimulating the cfFSH-R. Although the cfFSH-R can be considered as a promiscuous gonadotropin receptor, as it binds both types of fish gonadotropins (Bogerd *et al.* 2001), it showed a slight preference for rcfFSH over rcfLH. A similar preference for FSH-type gonadotropins was also observed when testing salmon and trout gonadotropins on this receptor (Schulz *et al.* 2001). Interestingly, the receptor type in coho salmon gonads which, based on its localization on

granulosa and Sertoli cells has been suggested to be an FSH-R-like receptor, showed similar ligand-binding properties, i.e. both gonadotropins were bound but a preference for FSH was observed (Yan *et al.* 1992, Miwa *et al.* 1994). rcfFSH was also able to activate the cfLH-R, albeit with a much lower efficacy (>300-fold EC_{50}) than the rcfLH and purified, pituitary-derived cfLH. Within the concentration range analyzed, however, rcfFSH had comparable bioactivities to both types of cfLHs in stimulating testicular steroidogenesis. This discrepancy indicates that the cfFSH-stimulated steroidogenesis may be mediated via the cfFSH-R, either directly by the FSH-R being expressed on the Leydig cells, although such an expression pattern has not been described yet in vertebrates, or alternatively via paracrine effects of Sertoli cell-derived factors (Lejeune *et al.* 1996). Similarly, salmon FSH has also been shown to be steroidogenic, and able to activate both the amago salmon FSH-R and LH-R (Planas & Swanson 1995, Oba *et al.* 1999*a*,*b*).

In summary, bioactive rcfLH and rcfFSH have been produced using the *Dictyostelium* expression system. Since repeated attempts to purify cfFSH from catfish pituitaries derived from different developmental stages were unsuccessful, the availability of recombinant hormone allowed us to obtain the first functional data on cfFSH. rcfFSH is steroidogenic, despite an ~1000-fold higher affinity for the cfFSH-R than for the cfLH-R, whereas rcfLH activates both the cfLH-R and cfFSH-R with a comparable efficacy. In order to elucidate the gonadotropin-mediated mechanism(s) regulating testis function in African catfish in more depth, we are presently determining the spatio-temporal expression patterns of both cfFSH-R and cfLH-R in the testis and of the cfFSHβ-subunit in the pituitary, as well as generating a cfFSH-specific antibody to develop a cfFSH-specific RIA.

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